Mn(II)-Oxidizing Bacteria Are Abundant And Environmentally Relevant Members Of Ferromanganese Deposits In Caves Of The Upper Tennessee River Basin

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Abstract

The upper Tennessee River Basin contains the highest density of our nation's caves; yet, little is known regarding speleogenesis or Fe and Mn biomineralization in these predominantly epigenic systems. Mn:Fe ratios of Mn and Fe oxide-rich biofilms, coatings, and mineral crusts that were abundant in several different caves ranged from ca. 0.1 to 1.0 as measured using ICP-OES. At sites where the Mn:Fe ratio approached 1.0 this represented an order of magnitude increase above the bulk bedrock ratio, suggesting that biomineralization processes play an important role in the formation of these cave ferromanganese deposits. Estimates of total bacterial SSU rRNA genes in ferromanganese biofilms, coatings, and crusts measured approximately 7×107–9×109 cells/g wet weight sample. A SSU-rRNA based molecular survey of biofilm material revealed that 21% of the 34 recovered dominant (non-singleton) OTUs were closely related to known metal-oxidizing bacteria or clones isolated from oxidized metal deposits. Several different isolates that promote the oxidation of Mn(II) compounds were obtained in this study, some from high dilutions (10–8–10–10) of deposit material. In contrast to studies of caves in other regions, SSU rRNA sequences of Mn-oxidizing bacterial isolates in this study most closely matched those of Pseudomonas, Leptothrix, Flavobacterium, and Janthinobacterium. Combined data from geochemical analyses, molecular surveys, and culture-based experiments suggest that a unique consortia of Mn(II)-oxidizing bacteria are abundant and promoting biomineralization processes within the caves of the upper Tennessee River Basin.

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Introduction

Within the continental United States, carbonate bedrock underlies 20% of the land area east of the Mississippi River, forming an intricate karst hydrologic network with numerous caves (Christman and Culver 2001; White et al. 1995). As such, the southern Appalachians contain numerous caves: of the 50,000 cave systems known to exist in the United States, ca. 14% occur within the state of Tennessee (Barton and Jurado 2007). However, to date only a few studies describe the microbial communities of Appalachian cave systems (Angert et al. 1998; Campbell et al. 2011; Engel et al. 2001; Shapiro and Pringle 2010; Simon et al. 2003), and none of these address in a significant way the geomicrobiology of an epigenic cave system, which is the primary method of speleogenesis in cave and karst systems worldwide (White et al. 1995).

Ferromanganese deposits are quite common in caves in the southern Appalachians. However, our knowledge of the microbial consortia associated with cave ferromanganese deposits is limited to research conducted in cave systems located in the southwest United States (Cunningham et al. 1995; Northup et al. 2003; Spilde et al. 2005, 2006), which formed via hypogene speleogensis (characteristically deeper cave formation via sulfuric acid in ascending groundwater) rather than epigene speleogensis (typically shallow cave formation via carbonic acid in descending meteoric water). Therefore questions remain unanswered regarding 1) the nature of these shallow, epigenic cave systems in the southern Appalachians, 2) how similar these shallow cave systems are to their deeper, hypogene counterparts and 3) to what extent surface waters and biota influence microbial consortia and biomineralization processes within shallow cave systems. Answers to these questions require basic exploratory efforts in order to generate hypotheses that provide a framework and direction for future research

endeavors. This point is particularly salient regarding research within understudied environments, such as the cave systems of the southern Appalachians.

The formation of cave mineral deposits and speleothems was long thought to be controlled primarily by abiotic processes as a result of microsite environmental conditions (e.g., temperature, pH, solution chemistry), and changes in redox conditions (Barton and Northup 2007; Engel et al. 2004; Northup and Lavoie 2001). However, more recent geomicrobiology research lends support to the hypothesis that microbes play a role in the formation and dissolution of cave mineral deposits via direct and indirect metabolic activities and biomineralization processes (Barton and Luiszer 2005; Cañaveras et al. 2006; Jones 2001; Melim et al. 2001; Northup et al. 1997; de los Ríos et al. 2011; Spilde et al. 2005; Taboroši 2006). In particular, microbial reactions have been shown to promote the formation of cave manganese oxide and ferromanganese (mixed Fe and Mn oxides) deposits, such as crusts (Northup et al. 2000, 2003; Spilde et al. 2005), manganese flowstones (Gradziński et al. 1995), rock coatings (Allouc and Harmelin 2001; Peck 1986), and manganese stromatolites (Rossi et al. 2010).

Cave ferromanganese deposits may contain Mn oxide, Mn hydroxide, and Mn oxyhydroxide minerals (collectively referred to hereafter as Mn oxides), and the mineralogy of these deposits can be quite complex (Onac and Forti 2011; Post 1999; White et al. 2009). The oxidation of Mn(II) to Mn(III) or Mn(IV) is kinetically inhibited in the absence of a catalyst at near-neutral pH of most environments. Microorganisms are known to catalyze the oxidation of Mn(II) compounds, increasing reaction rates up to five orders of magnitude relative to abiotic oxidation rates (Nealson et al. 1988; Dixon and Skinner 1992; Francis and Tebo 2002). Therefore, rapid Mn(III/IV) oxide depositional rates, especially those which exceed predicted abiotic reaction rates in a given environment, are a strong indication of microbial involvement in deposit formation (Nealson et al. 1988).

Mn oxide minerals have highly charged surfaces and are biogeochemically active, demonstrating the ability to degrade humic substances (Sunda and Kieber 1994), scavenge reactive oxygen species (Archibald and Fridovich 1981; Daly et al. 2004; Ghosal et al. 2005; Learman et al. 2011), concentrate rare earth elements (Onac et al. 1997), and influence trace metal bioavailability (Nelson et al. 1999; Post 1999; Kay et al. 2001; Manceau et al. 2002; Villalobos et al. 2005; Toner et al. 2006) and speciation (Fendorf et al. 1992; White et al. 2009). Biogenic oxides, which tend to have higher percentages of vacancies and smaller particle sizes (Learman et al. 2011; Webb et al. 2005), demonstrate an increased sorptive capacity relative to abiotically produced oxides (Nelson et al. 1999). Therefore, biogenic Mn oxides may exert a greater impact on local geochemistry than abiotically generated deposits.

In this study, we examined the geomicrobiology of ferromanganese deposits in an epigene cave system located in the cave-rich but poorly studied southern Appalachian karst region. Using a combination of molecular-based SSU rRNA analysis and culture-based methodologies, we demonstrate that several different species of Mn(II)-oxidizing bacteria are abundant and promoting biomineralization processes within the caves. Molecular evidence from the primary study site, Carter Saltpeter Cave, suggests that the Mn-oxidizing microbial consortia in ferromanganese deposits within shallow cave systems harbor a unique signature when compared to similar deposits in deep cave systems located within the southwest United States.

Methods

Field Description

The study area (Figure 1, inset) is comprised of several epigenic caves, all located in the Ordovician Knox Group (Oder 1934) within the upper Tennessee River Basin. The primary study site, Carter Saltpeter Cave (Carter County, TN, Figure 1), herein referred to as CSPC, is a shallow cave system at a depth of approximately 30 m. It is an epigenic cave system typical of those found within the Appalachian region, and evidence of anthropogenic impact is widespread throughout the system. Rockhouse Cave (R) is located less than 2 km east of CSPC, and the two systems are hydrologically connected (Gao et al. 2006a, 2006b). Worley's Cave, located 24 km northeast of CSPC in Sullivan County, TN, is frequently visited by humans and contains a subterranean creek system that exits the cave and flows to the south fork of the Holstein River. Recent work (Y. Gao, unpublished data) has demonstrated that Worley's cave is hydrologically connected to sinkholes in nearby farm fields and therefore may be susceptible to agricultural runoff. In contrast to these three anthropogenically impacted caves, Daniel Boone Caverns (herein referred to as DBC) in Scott County, VA is gated and access is controlled by the landowner. Therefore, this cave is rarely visited. DBC is located in an isolated forest location on the top of a ridge and is not subject to agricultural or municipal runoff. It contains several pools and drip networks, but does not have an extensive subsurface hydrologic system.

Sample Collection

Samples (Table 1) were collected periodically, in roughly three month intervals, from July 2009 to September 2011 in four cave systems. Ferromanganese deposits were identified as black/chocolate brown biofilms, coatings, or mineral-rich crusts that coat cave walls and speleothems. Deposits were screened for the presence of Mn oxides using 0.04% Leucoberbein Blue (LBB), a redox indicator that is oxidized by Mn(III) or Mn(IV) to produce a bright blue color change (Krumbein and Altmann 1973). LBB tests were conducted by scraping the deposit surface using Whatman chromatography paper, flushing the sample with LBB, and looking for the production of the color change described above. Deposit morphology was highly variable within systems (Figure 2), with LBB-positive samples collected from biofilms, ferromanganese coatings and crusts, and ferromanganous micronodules. Samples were collected aseptically by scraping the deposit surface, placed in a cooler on ice or dry ice and transported back to the lab for immediate plating/inoculation (for culturing), DNA extraction (for clone libraries and qPCR), or fixation (for microscopy).

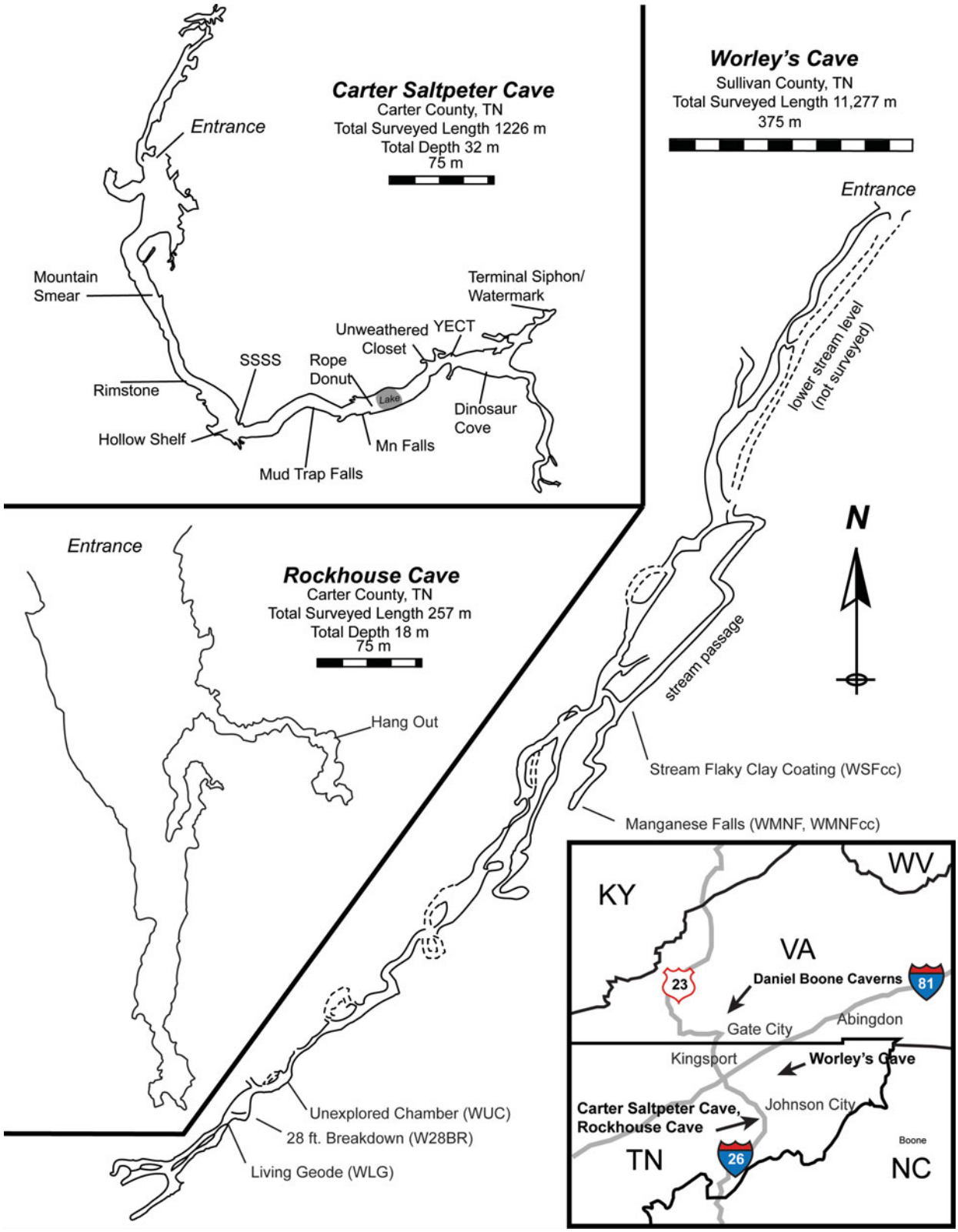

Fig. 1. Maps of the primary study sites: Carter Saltpeter Cave, Rockhouse Cave, and Worley's Cave. A map of Daniel Boone Caverns was not available at the time of this writing. Sampling locations are labeled with hash marks. Regional map inset shows the relative location of all four cave systems that are included in this study. Carter Saltpeter Cave survey conducted on February 8, 1981 by L. Adams, R. Knight, R. Page, and T. Wilson. Rockhouse Cave survey conducted on May 6, 1980 by L. Adams, T. Gingrich, and D. Nelms. Worley's Cave survey conducted from December 22, 1971 - August 8, 1973 by M. Adams, T. Anderson, C. Booth, R. Bowery, J. Cox, T. Harrison, D. Mire, A. Powers, D. Powers, and J. Powers. All cave maps adapted by S.K. Carmichael (color figure available online).

Table 1. A descriptive summary of samples obtained from cave ferromanganese deposits in this study

Sample	Ferromanganese Deposit Morphology	Analyses Conducted
Dinosaur Cove (CSPC)[a]	Coating	ICP-OES, qPCR, EM
Dinosaur Cove Mud (CSPC)[a]	Coating	ICP-OES, EM
Dinosaur Cove Popcorn (CSPC)[a]	Micronodule	ICP-OES, EM
Mn Falls (CSPC)[a]	Thick biofilm	Clone library construction, Cultures, ICP-OES, qPCR, EM
Mud Trap Falls (CSPC)	Coating	Clone library construction, Cultures, *in-situ* glass-slide cultivation, EM, qPCR
Watermark (CSPC)	Coating	ICP-OES, qPCR, EM
Top of V (DBC)	Coating	Cultures, *in-situ* glass-slide cultivation, EM
Hang Out (R)	Biovermiculation	qPCR
Dolomite w/ Crust (W)	Crust	qPCR
Mn Chamber (W)[a]	Crust	ICP-OES, EM
Ribbon Rock (W)[a]	Crust	ICP-OES, qPCR, EM
River Bank (W)[a]	Crust	ICP-OES
Weathered Ribbon Rock (W)[a]	Crust	ICP-OES, qPCR, EM

[a]Denotes a sample where the Mn:Fe ratio is enriched relative to the average Mn:Fe ratio for the bedrock in the region (Table 2).
Cave locations are abbreviated as follows: Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), Rockhouse Cave (R), and Worley's Cave (W). Analyses conducted are abbreviated as follows: Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES), real-time quantitative PCR (qPCR), and electron microscopy (EM).

Field samples for electron microscopy were fixed in the field using gluteraldehyde with sodium phosphate buffer.

Geochemistry and Biogeochemistry

A subset of cave ferromanganese samples were analyzed at Appalachian State University to determine substrate mineralogy using a Shimadzu 6000 powder X-ray diffractometer with a Cu X-ray source and measured from 5–80° 2θ. Species were identified using the PDF/4+ Minerals Database. A subset of samples were collected for metal analyses in December 2009 (Worley's Cave) and January 2010 (CSPC), lyophilized over a 48 hour period, then digested for metal analyses following US EPA SW846 Method 3051A: Microwave Assisted Acid Digestion of Sediments, Sludges, Soils, and Oils (Link et al. 1998; SW-846 EPA Method 3051A 2007). Elemental analysis was performed in duplicate on several rock, coating, and biofilm samples to determine total Mn and Fe content using a Varian 710-ES Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES).

Real-time Quantitative PCR

Ten samples from three cave systems were analyzed using real-time quantitative PCR to quantify the relative abundance of Archaea and Bacteria in the CSPC, Rockhouse, and Worley's cave systems. Primers were selected for each domain that demonstrated broad coverage over the 16S rRNA region. The forward primer 338F 5′-TCCTACGGGAGGCAGCAGT-3′ (Nadkarni et al. 2002) was paired with the reverse primer 518R 5′-ATTACCGCGGCTGCTGG-3′ (Einen et al. 2008) to target the bacterial 16S rRNA gene sequence. The primer pair 967F 5′-AATTGGCGGGGGAGCAC-3′ / 1060R 5′-GGCCATGCACCWCCTCTC-3′ (Cadillo-Quiroz et al. 2006) was selected to target the archaeal 16S rRNA gene sequence. Amplifications were performed in triplicate on an Applied Biosystems^{TM} 7300 Real-Time PCR System (Carlsbad, CA) using Maxima^{TM} SYBR Green/ROX qPCR Master Mix (Fermentas, Glen Burnie, MA) with 2 ng sample DNA/well. Calibration curves for quantification were generated using one of the following standards: 1) plasmid DNA containing the SSU rRNA gene from *Rhodobacter* sp. CR07–74 (Bacteria, range of 10^2–10^9 target copies/μL) or 2) genomic DNA extracted using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA) from *Methanoregula boonei* 6A8 (Archaea, range of 10^2–10^7 target copies/μL). Circular plasmid DNA standards have been reported to cause overestimation of sample cell number using quantitative PCR (Hou et al. 2010). To circumvent this potential issue, plasmid DNA standards were lineraized by restriction digest using BssHII (New England BioLabs, Ipswich, MA). rRNA operon copy numbers in bacterial cells are variable (Fogel et al. 1999) and change based on environmental conditions (Klappenbach et al. 2000). Therefore, SSU rRNA gene copy number was normalized in experimental results using the average copy number for Archaea (1.07 copies/cell) and Bacteria (4.08 copies/cell) as reported by the Ribosomal RNA Operon Copy Number Database (Klappenbach et al. 2001) in March, 2010.

Fluorescence Direct Counts

Fluorescence direct counts were performed on samples from Mn Falls and Mud Trap Falls (CSPC) to validate real-time quantitative PCR results. Upon receipt in the lab, 0.1 g (wet weight) of biofilm material was mixed 1:10 w/v with 0.1% (final concentration) sodium pyrophosphate ($Na_4P_2O_7.10\ H_2O$) and vortexed for ten minutes to disrupt cell clumps and homogenize the material. Samples were fixed in a 4%

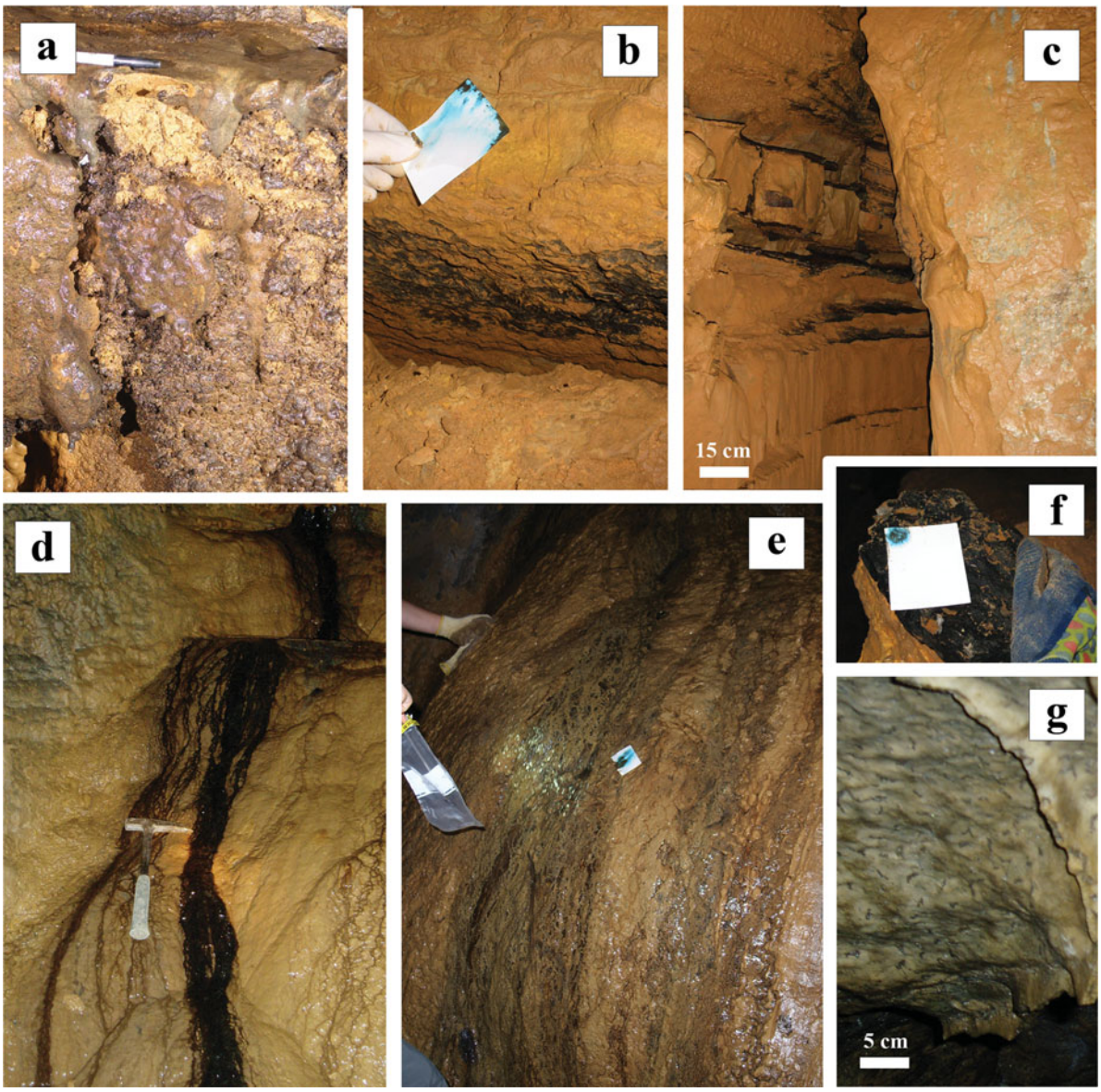

Fig. 2. Examples of ferromanganese deposit types found in the upper Tennessee River Valley karst network: a) coatings on cave coral at Dino Cove (CSPC); b) coatings on massive nontronite clay deposits (DBC); c) coatings on 5 cm thick calcite layers within cave walls (DBC); d) biofilm associated with a groundwater seep at Mn Falls (CSPC); e) coating associated with a groundwater seep at Mud Trap Falls (CSPC); f) coatings on calcite layers within cave walls show LBB+ signature (DBC); and g) biovermiculations (R). Cave systems are abbreviated as follows: Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), and Rockhouse Cave (R) (color figure available online).

paraformaldehyde solution and stored at 4°C overnight. Samples were then re-suspended by vortexing, and a 5 μL sample was then applied to a slide and evenly spread over 484 mm^2 surface area. Samples were stained with 1 μg/mL (final concentration) DAPI (4,6-diamino-2-phenylindole), and Citiflour Antifadent Mounting Medium AF1 (Electron Microscopy Sciences, Hatfield, PA) was applied to prevent bleaching of the DAPI fluorescent signal. Fluorescence direct counts were conducted at 100X magnification on an Olympus Bx51 fluorescence microscope. Fields of view were randomly selected and counted until a minimum of 300 cells/sample were visualized and recorded.

Bacterial and Archaeal Clone Library Construction

DNA was extracted from two LBB-positive cave deposits, Mn Falls and Mud Trap Falls, located within the primary study site (CSPC) using a bead beating protocol with the Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, OH). The concentration of extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracted DNA was used to create a total of four clone libraries. For Bacteria, one library each was created from DNA extracted from light and dark material at the Mn Falls site in CSPC. This site appeared to be highly

active as it was strongly LBB positive and had streamers of Mn-oxide-rich biofilm material flowing down the cave wall at the time of sampling. Both dark and light material was sampled in attempt to detect a significant enrichment in one or more groups that may be implicated in Mn(II)-oxidation.

Small clone libraries were constructed since the main goal was to evaluate the most abundant OTUs, rather than rare OTUs, in each sample. Approximately 3 ng of environmental DNA was used as a template for the PCR amplification of bacterial DNA using the primer 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ (Lane 1991) combined with a modified version of 1492R primer 5′-RGYTACCTTGTTACGACTT-3′ (for Bacteria) (Emerson and Moyer 2002). Each 25 μL PCR reaction contained, in final concentrations, 1.25 U AmpliTaq Gold (Applied Biosystems, Carlsbad, CA), 200 μM each primer, 1X PCR Gold Buffer (Applied Biosystems, Carlsbad, CA), 2 mM $MgCl_2$ Solution (Applied Biosystems, Carlsbad, CA), 200 μM each dNTP, and 2X BSA (New England Biolabs, Ipswich, MA). A MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA) was used for all PCR-amplification reactions. The amplification protocol for bacterial clone libraries was as follows: an initial denaturation of 95°C for 10 min, followed by 30 cycles of 94°C for 5 min, 64°C for 90 s, 72°C for 3 min, and a final extension of 72°C for 7 min. PCR amplifications were conducted in triplicate, and amplified PCR products were pooled before purification using a Montage® PCR Purification Kit (Millipore, Billerica, MA).

For Archaea, one library was created from DNA extracted from the Mn Falls and Mud Trap Falls sites. Approximately 3 ng of environmental DNA was used as a template for the PCR amplification of archaeal DNA using the using the 109F 5′-ACKGCTCAGTAACACGT-3′ and 912R 5′-CTCCCCCGCCAATTCCTTTA-3′ primer pair for Archaea (Lueders and Friedrich 2000). Each 25 μL PCR reaction contained, in final concentrations, 1.25 U AmpliTaq Gold (Applied Biosystems, Carlsbad, CA), 200 μM each primer, 1X PCR Gold Buffer (Applied Biosystems, Carlsbad, CA), 2 mM $MgCl_2$ Solution (Applied Biosystems, Carlsbad, CA), 200 μM each dNTP, and 2X BSA (New England Biolabs, Ipswich, MA). The amplification protocol for archaeal clone libraries is as follows: an initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 90 s, and a final extension of 72°C for 6 min. PCR amplifications were conducted in triplicate, and amplified PCR products were pooled before purification using a Montage® PCR Purification Kit (Millipore, Billerica, MA).

PCR products were cloned into TOPO TA *pcr*®2.1 vectors (Invitrogen, Carlsbad, CA), and plasmid DNA extracted from transformants using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was screened by sequencing using the M13F (-20) primer. 96 well plates of glycerol stocks were prepared for each sample site using each primer set and sequenced using M13F(-20) and M13R(-27) primers. Sequencing was conducted using a Sanger platform at Beckman-Coulter Genomics (Danvers, MA). Chimeric sequences were eliminated from analysis prior to consensus sequence construction. Sequences from both bacterial and archaeal libraries were pooled (creating a ca. 180 sequence bacterial library and 65 sequence archaeal library) for DOTUR analysis in order to make OTU determinations (Schloss and Handelsman 2005). Representative sequences for each OTU for Archaea or each dominant OTU (as defined by two or more sequence representatives) for Bacteria were chosen based on sequence length and quality. For the bacterial dominant OTUs, additional sequencing of transformant plasmid DNA was conducted using primers 357F 5′-CCTACGGGAGGCAGCAG-3′, 926R 5′-CCGYCWATTCMTTTRGT TT-3′, and 1098R 5′-GGGTYKCGCTCGTTGC-3′ to obtain full-length (ca. 1500 bp) SSU rRNA gene sequences. Contigs were assembled using Sequencher sequence analysis software (Version 5.0, Build 7082, Gene Codes Corporation, Ann Arbor, MI). Good's nonparametric coverage was estimated using the equation [1-(n/N)] x 100, where "N" is the total number of clones evaluated and "n" is the number of singleton OTUs (Good 1953).

For phylogenetic analysis, additional sequences of interest were selected using ARB (Ludwig et al. 2004) and the NCBI taxonomic database (Johnson et al. 2008). OTU and additional sequences of interest were aligned using the on-line SILVA aligner (Pruesse et al. 2007). Phylogenetic trees were constructed using the PHYLIP software package (Felsenstein 2004) by conducting both neighbor-joining and maximum likelihood analysis. Clone sequences of archaeal OTUs and dominant bacterial OTUs were deposited in GenBank under the accession numbers JN820160-JN820219.

Isolation of Mn(II)-oxidizing Microorganisms on Agar Media

Mn-oxide-rich samples from ferromanganese biofilms, micronodules, and coatings on rock walls and speleothems were collected in an attempt to cultivate Mn(II)-oxidizing cave microorganisms. Samples were transported to the lab on ice and immediately plated on a variety of media designed to target the phylogenetically diverse array of Mn-oxidizers. A modified version of AY media (Santelli et al. 2011) was created by supplementing the media post-autoclaving with 100 μM $MnCl_2$. A modified version of Burk's nitrogen-free medium (Mohandas 1988) was created to target putative nitrogen-fixing, Mn(II)-oxidizing microorganisms by substituting an equimolar concentration of succinic acid, disodium salt for sucrose as a carbon source and amending the media (post-autoclaving) with 100 μM $MnCl_2$ and 3.7 mM $FeCl_3$. A novel media, Nitrate Mineral Salts (NMS), was designed for this study to target methylotrophic Mn(II)-oxidizers. NMS contains (in g L^{-1}) 1 $MgSO_4 \cdot 7H_20$, 0.14 $CaCl_2 \cdot 2H_20$, 1 KNO_3, 0.27 KH_2PO_4, 0.3 NaH_2PO_4, 1 mL trace element solution (containing in mg L^{-1} 1000 EDTA, 400 $FeSO_4 \cdot 7H_20$, 250 $CuSO_4 \cdot 5H_20$, 20 $ZnSO_4 \cdot 7H_20$, 6 $MnCl_2 \cdot 4H_20$, 60 H_3BO_3, 40 $CoCl_2 \cdot 6H_20$, $2CaCl_2 \cdot 2H_20$, 4 $NiCl_2 \cdot 6H_20$, 6 $Na_2Mo_4 \cdot 2H_20$). pH of the media was adjusted ca. 7.1–7.2 before autoclaving, and 15 g agar was added for plates. NMS media was supplemented post-autoclaving with sterile 0.02 M Hepes buffer pH 7.2, 5 μM ferrous ammonium citrate, 0.2% v/v vitamin solution for J medium (Tebo et al. 2007), and 100 μM $MnCl_2$. For agar plates, a 0.05% v/v methanol was added as a carbon source; a 50:50 CH_4(g):air mix was used as

the sole carbon source for liquid media. A new medium, FMO2, was designed for this study by S.L. Bräuer and contains (in g L^{-1}) 10 mL Major Metals 1 solution (containing in g L^{-1} 12 NaCl, 1.2 KCl, 5 $MgCl_2 \cdot 6H20$, 1 KH_2PO_4, 2 NH_4Cl, 1 $CaCl_2 \cdot 2H_20$), 1 mL 1000X Trace Metal 1 Solution with NTA (containing in g L^{-1} 0.15 $CoCl_2 \cdot 6H_20$, 0.15 $ZnCl_2$, 0.05 H_3BO_3, 0.02 $NiCl_2 \cdot 6H_20$, 0.01 $Na_2Mo_4 \cdot 2H_20$, 0.4 $FeCl_2 \cdot 4H_20$, 0.1 $MnSO_4 \cdot 4H_20$, 3 $MgSO_4 \cdot 7H_20$, 0.1 $CaCl_2 \cdot 2H_20$, 0.01 $CuSO_4 \cdot 5H_20$, 0.18 $AlK(SO_4)_2 \cdot 12H_20$, 1.5 NTA), and 0.05 yeast extract. pH of the media was adjusted to ca. 7.0–7.2 before autoclaving, and either 15 g agar or Gelrite gellan gum [an alternative solidifying agent (Hara et al. 2009)] was added for plates. The media was supplemented post-autoclaving with sterile 0.02 M Hepes buffer pH 7.2, 5 μM ferrous ammonium citrate, 0.2% v/v vitamin solution for J medium (Tebo et al. 2007), 100 μM $MnCl_2$, and either 10 mM arabinose, 10 mM succinate, or 0.05% casamino acids as a carbon source (final concentrations). Plates were inoculated by spreading 80 μL of a 1% v/v Mn oxide rich sample in 0.02M Hepes buffer pH 7.2 on agar-solidified media. All cultures were incubated in the dark at 10°C to mimic environmental conditions within caves. Some plates were incubated under full oxygen conditions, while others were incubated microaerophilically (roughly 10% atmospheric air in a sealed environment), with the recognition that oxygen limited environments constitute important niches in cave systems (Portillo and Gonzalez 2009). Mn(II)-oxidation was confirmed in isolates by LBB testing; LBB-positive isolates were re-streaked for isolation a minimum of three times on the equivalent growth medium.

Serial Dilutions

Serial dilutions to extinction were conducted in Cellstar 96-well culture plates (greiner bio-one, Monroe, NC) using three different media types: FMO2 media with either 10% 2M arabinose, 10% 2M succinate, or 10% casamino acids as a carbon source. Inocula from the most dilute sample that grew and produced dark brown/black precipitates was transferred to the equivalent agar-solidified growth medium and were re-streaked for isolation a minimum of three times. Mn-oxidation was confirmed in isolates by LBB testing. All cultures were incubated in the dark at 10°C, mimicking environmental conditions within caves.

Identification of Isolates

Once a colony was isolated, a colony PCR reaction was used to screen the microorganism for phylogenetic placement using the universal bacterial primer 357F 5′-CCTACGGGAGGCAGCAG-3′. For screening, each 25 μL PCR reaction contained final concentrations of 1X PCR Master Mix (Fermentas, Glen Burnie, MD) and 0.2 μM each primer (27F and a modified version of 1492R, detailed above). DNA was added to each reaction by touching an isolated colony with a sterile pipette tip and washing the tip in the reaction mixture. The amplification protocol for screening isolates is as follows: an initial denaturation of 95°C for 10 min, followed by 30 cycles of 94°C for 1 min, 55°C for 90 sec, 72°C for 3 min, and a final extension of 72°C for 7 min. Isolates of interest were cloned as previously described using TOPO TA *pcr*®2.1 vectors (Invitrogen, Carlsbad, CA), and plasmid DNA containing the SSU rRNA gene sequence was sequenced using M13F(-20), M13R(-27), 357F 5′-CTACGGGAGGCAGCAG-3′, 926R 5′-CCGYCWATTCMTTTRAGTTT-3′, and 1098R 5′-GGGTYKCGCTCGTTGC-3′ primers to obtain a full-length (ca. 1500 bp) SSU rRNA gene sequence for phylogenetic placement as described above. Contigs were assembled using Sequencher sequence analysis software (Version 5.0, Build 7082, Gene Codes Corporation, Ann Arbor, MI). Isolate sequences were deposited in GenBank under the accession numbers JN820147-JN820159.

Detection of in-situ Microbial Mn-oxidation with Scanning Electron Microscopy

Environmental samples were examined using scanning electron microscopy (SEM) to visualize microbial morphologies associated with ferromanganese deposits. In addition to direct sampling of rock or sediment substrate, polycarbonate 0.45 μm filters were affixed to glass slides with dots of plumber's glue and set in various locations in CSPC for a period of six months or more. Upon collection, slides and substrate samples were placed in a sterile falcon tube and preserved in a 2.5% gluteraldehyde mixture, then dehydrated by soaking the filters in a series of 50%, 75%, and 85% ethanol in water solutions, followed by soaking two times in 100% ethanol. Samples were treated for a minimum of two hours in each ethanol solution. Samples were then critically point dried using liquid CO_2 in a Polaron critical point dryer and imaged using a FEI Quanta 200 Environmental Scanning Electron Microscope with an EDAX Genesis XM energy dispersive X-ray spectrometer for elemental analysis.

Transmission Electron Microscopy and Elemental Analysis

Mn-oxidizing bacterial cultures were examined using a JEOL JEM-1400 transmission electron microscope (TEM) equipped with an Oxford INCA energy dispersive X-ray detector (EDS) to confirm the presence of Mn oxides associated with bacterial cells. Samples were mounted on Formvar Carbon Type-B, 200 mesh Cu TEM grids (Ted Pella, Redding, CA) by diluting liquid cultures 1:5 using sterile deionized water and applying 5 μL dilution to each grid. TEM grids were allowed to air dry in a laminar flow cabinet. This process was repeated a total of three times, with a total volume of 15 μL of diluted culture applied to each grid, and then carbon coated. Samples were initially imaged using transmission electron microscopy and spot analyzed with EDS to confirm the presence of Mn deposits. Several samples were selected for elemental mapping via scanning transmission electron microscopy (STEM) to confirm the locations of Mn within the sample. In all cases, EDS spot analysis (data not shown) demonstrated the presence of concentrated Mn deposits associated with bacterial cells.

Table 2. Biogeochemical analyses of ferromanganese deposits located in cave systems within the upper Tennessee River Basin

Sample Site	Mn (ppm)	Fe (ppm)	Mn:Fe Concentration	Substrate Geochemistry
Ribbon Rock (W)	284.6	266.8	1.07	Quartz, illite, trace dolomite
River Bank (W)	123.2	275.7	0.45	Quartz, orthoclase, illite, trace dolomite
Weathered Ribbon Rock (W)	125.4	323.8	0.39	Quartz, orthoclase, illite
Mn Chamber (W)	77.4	342.6	0.22	Quartz, trace calcite, minor orthoclase
Dinosaur Cove Popcorn (CSPC)	22.3	124.8	0.18	Calcite
Dinosaur Cove Mud (CSPC)	205.3	343.8	0.59	Nontronite
Watermark (CSPC)	14.3	254.7	0.06	Nontronite
Mn Falls (CSPC)	327.5	361.4	0.91	Nontronite
Dinosaur Cove (CSPC)	10.9	50.1	0.22	Calcite

Mn:Fe concentrations at sample sites were determined by ICP-OES analysis and are reported as an average. Substrate geochemistry, as determined by X-ray diffraction, is reported when known. Location of sample site is abbreviated below as follows: Worley's Cave (W), and Carter Saltpeter Cave (CSPC).

Results

Geochemical and Biogeochemical Analyses

All four cave systems (CSPC, Worley's, Rockhouse, and DBC, Figure 1) contain a variety of speleothem formations (flowstone, dripstone, soda straws, corrosion residue, Figure 2) and are particularly enriched in ferromanganese oxide deposits. The likely source of Fe(II) and Mn(II) necessary for the formation of these deposits is the bedrock dolomite of the Knox Dolomite Supergroup. The Knox Dolomite is a limestone, dolomite, and shale sequence with dolomite containing ca. 88–445 ppm Mn and ca. 1340–7050 ppm Fe, and typical Mn:Fe ratios of 0.001–0.256 (Montañez 1994).

Weathering and corrosion of the Knox Dolomite bedrock by infiltration of meteoric water containing carbonic acid produces a nontronite (smectite-type) clay residuum in addition to calcite speleothems in all four study sites, including flowstones, rimstones, and popcorn-like micronodules (cave coral). We observe ferromanganese crusts, coatings, and/or biofilms occurring on all substrate types. Geochemical analyses show that the concentration of total Mn in these crusts, coatings, and biofilms ranges from 10.9 ppm–327.5 ppm (Table 2). The Mn:Fe ratios in cave ferromanganese deposits vary between cave systems and within cave deposits, ranging from 0.06–1.07 (Table 2). However, no obvious trends have emerged that indicate a possible effect of substrate mineralogy or water content on Mn:Fe ratios in these deposits. For example, samples taken from different locations with similar substrate compositions in Carter Saltpeter Cave show both the second highest (0.91) and lowest (0.06) Mn:Fe ratios. Notably, the Mn:Fe ratio at all sample sites, with the exception of CSPC Watermark (Table 2), was enriched relative to the average Mn:Fe ratio for Knox Group rocks in that region (ca. 0.07) as reported by Montañez (1994). At sites where the Mn:Fe ratio approaches 1 (e.g. Ribbon Rock and Mn Falls), this would represent nearly an order of magnitude increase above the bulk bedrock ratio.

Quantification of Bacterial and Archaeal Abundance

Our qPCR results (Figure 3) demonstrated that total bacterial SSU rRNA genes in Mn oxide-rich samples from Mn Falls and Mud Trap Falls (CSPC- Figures 2d and 2e) represented approximately 9×10^9 cells/g wet weight, a number that was confirmed via fluorescence direct cell counts. Total bacterial SSU rRNA genes from other ferromanganese coatings and crusts (Figure 3) were up to two orders of magnitude lower, containing an estimated 7×10^7–2×10^9 cells/g wet weight. Archaeal SSU rRNA gene sequences from cave samples were estimated to represent from 5×10^6–1×10^8 cells/g wet weight, with an average of 2.7×10^7 cells/g wet weight in biofilm samples vs. 3.8×10^7 cells/g wet weight in ferromanganese coatings and crusts. Archaeal percentages were highest at several sample sites within the CSPC and Worley's cave systems: Dino Cove (ca. 7%), Ribbon Rock (ca. 6%), Watermark (ca.

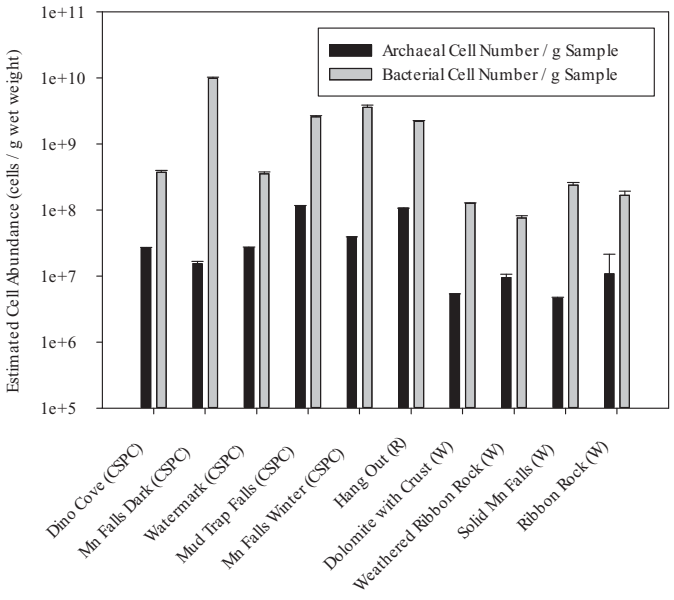

Fig. 3. Real-time qPCR quantification of SSU rRNA gene copy number of total Archaea and Bacteria in samples from Carter Saltpeter (CSPC), Rockhouse (R), and Worley's (W) Caves. SSU rRNA gene copy number was normalized by 1.77 copies (for Archaea) and 4.08 copies (for Bacteria) to obtain estimates of cell abundance. Analyses were conducted in triplicate and error bars represent standard error.

7%), and Weathered Ribbon Rock (ca. 11%). Overall, bacterial SSU rRNA gene copies represented on average 95% (89–99%) of the total estimated microbial (bacterial and archaeal) cell numbers at each site, while those of Archaea represented on average 5% (0.2%–11%).

Bacterial and Archaeal Community Structure

In this study, a SSU rRNA clone library survey was conducted on two ferromanganese-rich deposits located in close physical proximity within the CSPC system, Mn Falls (Table 2, Figure 2d, OTUs from this sample are prefaced by a *F* in Figure 4 and Figure 5) and Mud Trap Falls (Figure 2e, OTUs from this sample are prefaced by a *T* in Figure 4 and Figure 5). Using 98% sequence similarity to define archaeal operational taxonomic units (OTUs), analysis of archaeal libraries revealed 26 unique OTUs out of 65 total sequences from the Mn and Mud Trap Falls sites. Rarefaction analysis of sequencing data indicated the development of an asymptotic trend (data not shown); therefore, sampling efforts were sufficient in capturing the archaeal diversity within the microbial community. Good's nonparametic coverage estimator indicated approximately 66–75% coverage in the archaeal libraires.

Thaumarchaeal sequences were binned into 3 operational taxonomic units (OTUs/species) (Figure 4), representing ca. 40% of the total archaeal sequences in cave biofilm clone libraries. According to top BLAST hits, these OTUs were related to clone sequences from a variety of environments, from sediment, to the deep subsurface, freshwater systems, and other ferromanganese deposits. Thaumarchaeal OTUs represented members of the Marine Group 1 Thaumarchaea and SAGMA Groups 1 and 2. The dominant Thaumarchaeal sequence in the present study was OTU TDO2, sharing 98% identity over a 765 bp alignment to a clone sequence isolated from freshwater ferromanganous micronodules and sediments by Stein et al. (2001). Euryarchaeal sequences from clone libraries were binned into 23 OTUs (Figure 5), representing ca. 60% of the total archaeal sequences in the clone library data. OTUs were closely related to other clones isolated from soil, freshwater and marine systems, and low-temperature environments. Members of the Deep Sea Hydrothermal Vent Group 6 (DSHV6) represented 29% of the total archaeal sequence types in cave biofilm clone libraries, and 45% of the Euryarchaeal sequences.

Bacterial community composition in the CSPC system was much more diverse than archaeal community composition, a pattern that is consistent with most environmental surveys, including caves (Chelius and Moore 2004; Macalady et al. 2006; Macalady et al. 2007; Northup et al. 2003). Using a 97% identity cutoff, there were 114 unique OTUs out of ca. 180 total sequences. Rarefaction analysis revealed no evidence of the development of asymptotic trend (data not shown), indicating that sampling efforts were not sufficient to measure the full extent of diversity within the biofilm communities. Good's nonparametric coverage estimator indicated approximately 53–57% coverage for the bacterial libraries. For phylogenetic analysis, data were further reduced into 34 dominant OTUs representing ca. 100 sequences (Figure 6, Figure 7, and Figure 8). A dominant OTU was defined as representing two or more sequences in the clone library, and it is important to note that this approach would result in an underestimation of diversity in the clone library as ca. 80 singleton OTUs were eliminated from downstream phylogenetic analysis.

Dominant bacterial OTUs from relatively small (ca. 96 sequences) clone libraries of a cave biofilm represented a diverse taxonomic array, with sequences from recovered clones in the libraries representing members of the *Bacteroidetes* (26%), *Betaproteobacteria* (20%), *Alphaproteobacteria* (15%), *Acidobacteria* (12%), *Gammaproteobacteria* (10%), *Verrucomicrobia* (7%), *Planctomycetes* (5%), *Chlorobi* (2%), and *Deltaproteobacteria* (2%). Several dominant bacterial OTUs identified in this study were related to clones and environmental isolates from freshwater and marine systems, sediment, contaminated ecosystems, other cave systems, and ferromanganese deposits. Some (ca. 10%) of the 34 dominant OTUs were related to known Mn-oxidizers such as *Leptothrix* [OTU BF2AO7, Figure 6, 100% identical over a 1,485 bp alignment to *Leptothrix cholodnii* SP-6 (Emerson and Ghiorse 1992)] and *Pseudomonas* (OTUs BF2B07 and BF2E03, Figure 6). In addition, some (ca. 11%) of the 34 dominant OTUs were related to known Fe-oxidizers (*Leptothrix* spp.) or clones isolated from oxidized iron deposits (OTUs BF2C07 and BF2C10, Figure 8). Combined, 21% of the recovered clone sequences represented by dominant OTUs were related to known metal-oxidizers or clones isolated from oxidized metal environments.

Isolation of Mn(II)-oxidizing Microorganisms

Six Mn(II)-oxidizing isolates obtained from CSPC ferromanganese deposits clustered within the Gammaproteobacterial subphylum and are near members of the genus *Pseudomonas*, a common group of known Mn(II)-oxidizing microorganisms (Table 3, Figure 6). Cultures N4, T4, T2, and N3 were isolated on a modified version of Burk's N-free media (Mohandas 1988), although we have not yet established if these isolates are capable of N_2 fixation. Isolate N3 is 97% identical over a 1,502 bp alignment to OTU BF2E03, and isolates N4 and T4 are 97% identical (over a 1,181 and 1,473 bp alignment, respectively) to OTU BF2B07. Isolate Mn Falls 11 is a close relative (99% identity over a 1,128 bp sequence alignment) of *Pseudomonas putida*, a model organism used in the study of the molecular mechanisms involved in Mn(II)-oxidation (Geszvain and Tebo 2010).

Two Mn(II)-oxidizing isolates obtained from ferromanganese deposits within CSPC clustered within the *Betaproteobacteria* (Table 3, Figure 6): *Janthinobacterium* sp. A6 and *Leptothrix* sp. G6. *Janthinobacterium* sp. A6 oxidizes Mn(II) in liquid culture, and appears to oxidize Mn extracellularly, since clumps of Mn oxides were loosely associated with cells (Figure 9). Isolate A6 was obtained from a serial dilution culture containing 2.5×10^{-8}g wet weight biofilm material. Isolate A6 oxidized Mn(II) intermittently during maintenance of the culture, with newly inoculated cultures periodically losing the capability to oxidize Mn(II). However, the sequenced culture was oxidizing Mn(II), as confirmed by LBB testing.

Cave isolate *Leptothrix* sp. G6 oxidizes Mn along the sheath (Figure 9). This organism is 99% identical over a

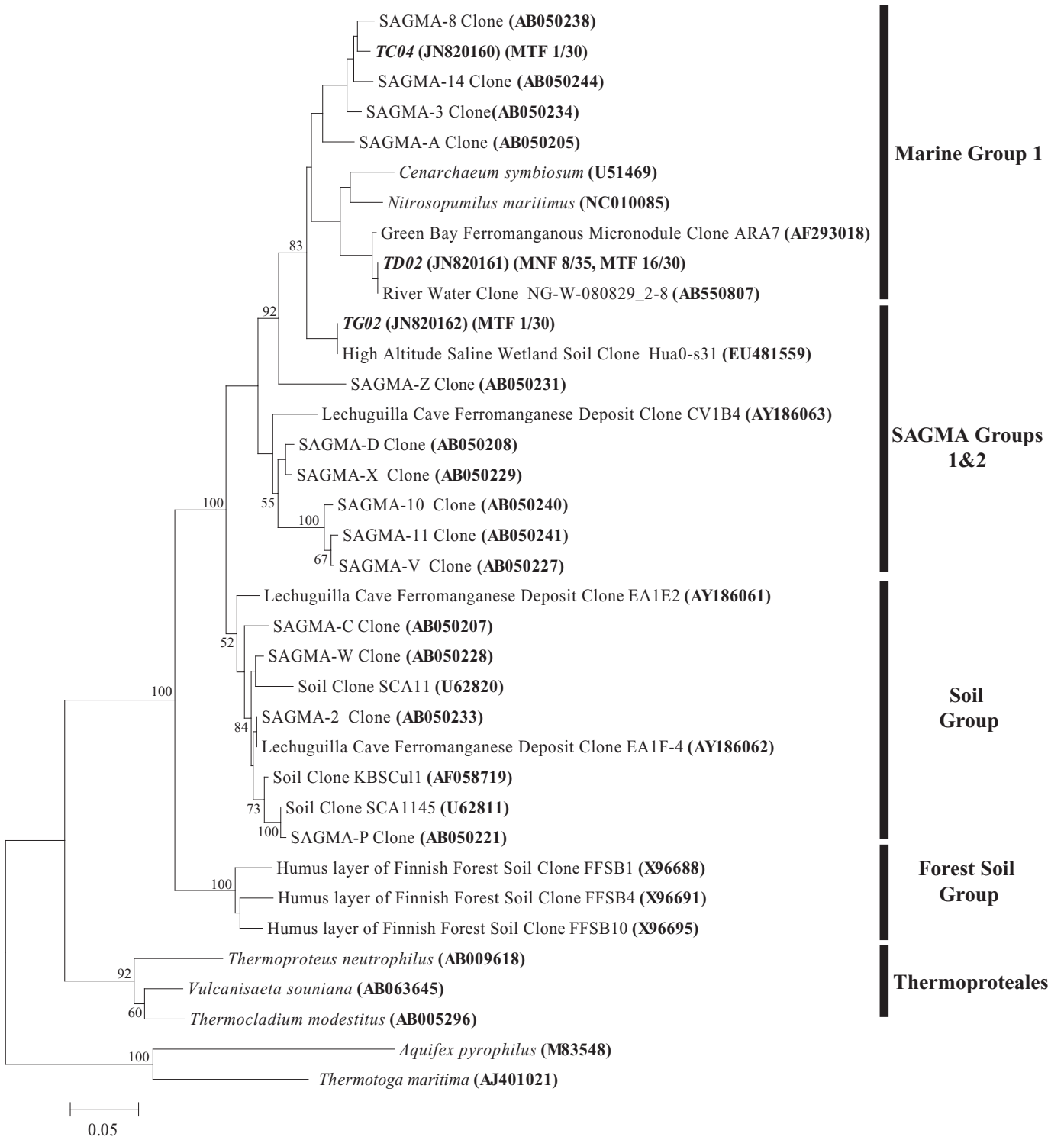

Fig. 4. Neighbor-joining tree based on Thaumarchaeal SSU rRNA gene sequences obtained from biofilms found in Carter Saltpeter Cave, Carter County, TN in this study. The number of sequences from each library [Mn Falls (MNF) and Mud Trap Falls (MTF)] representing a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position.

Fig. 5. Neighbor-joining tree based on Euryarchaeal SSU rRNA gene sequences obtained from biofilms found in Carter Saltpeter Cave, Carter County, TN in this study. The number of sequences from each library [Mn Falls (MNF) and Mud Trap Falls (MTF)] representing a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position.

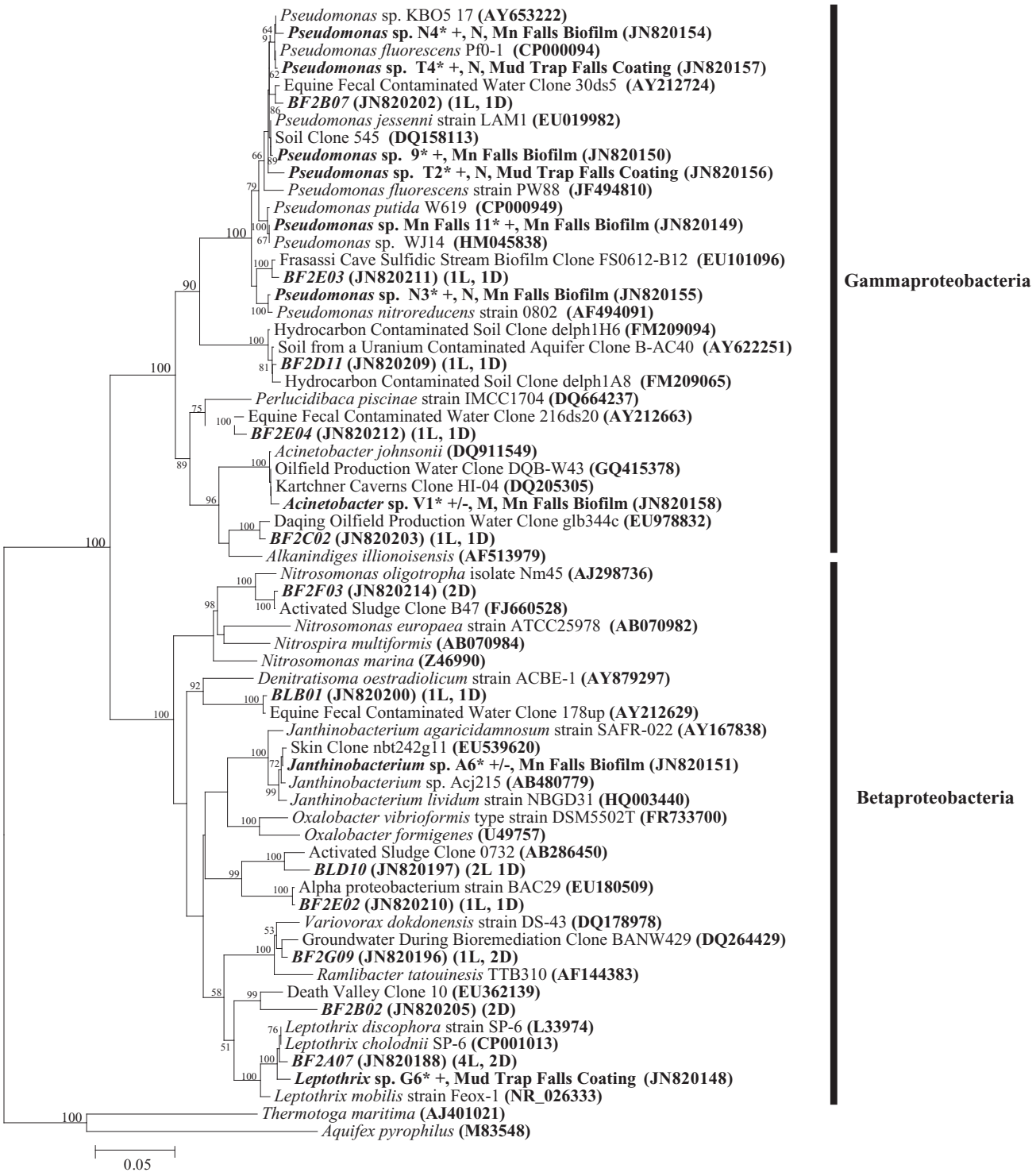

Fig. 6. Neighbor-joining tree inferring the phylogenetic relationship between ***cultured strains**** (asterisks) and those found in ***clone libraries*** in Carter Saltpeter Cave, Carter County, TN in this study for sequences clustering in the *Beta*- and *Gammaproteobacteria*. Isolates that oxidize Mn continually are indicated by a +; isolates that oxidize Mn intermittently are indicated by a ±. Putative Nitrogen-fixing, Mn(II)-oxidizers are indicated by an **N**; putative methylotrophic Mn(II)-oxidizers are indicated by an **M**. Source of isolation is noted immediately before accession number. The number of sequences from each library [Mn Falls Light (L) and Mn Falls Dark (D)] representing a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicated the expected number of changes per sequence position (see scale bar).

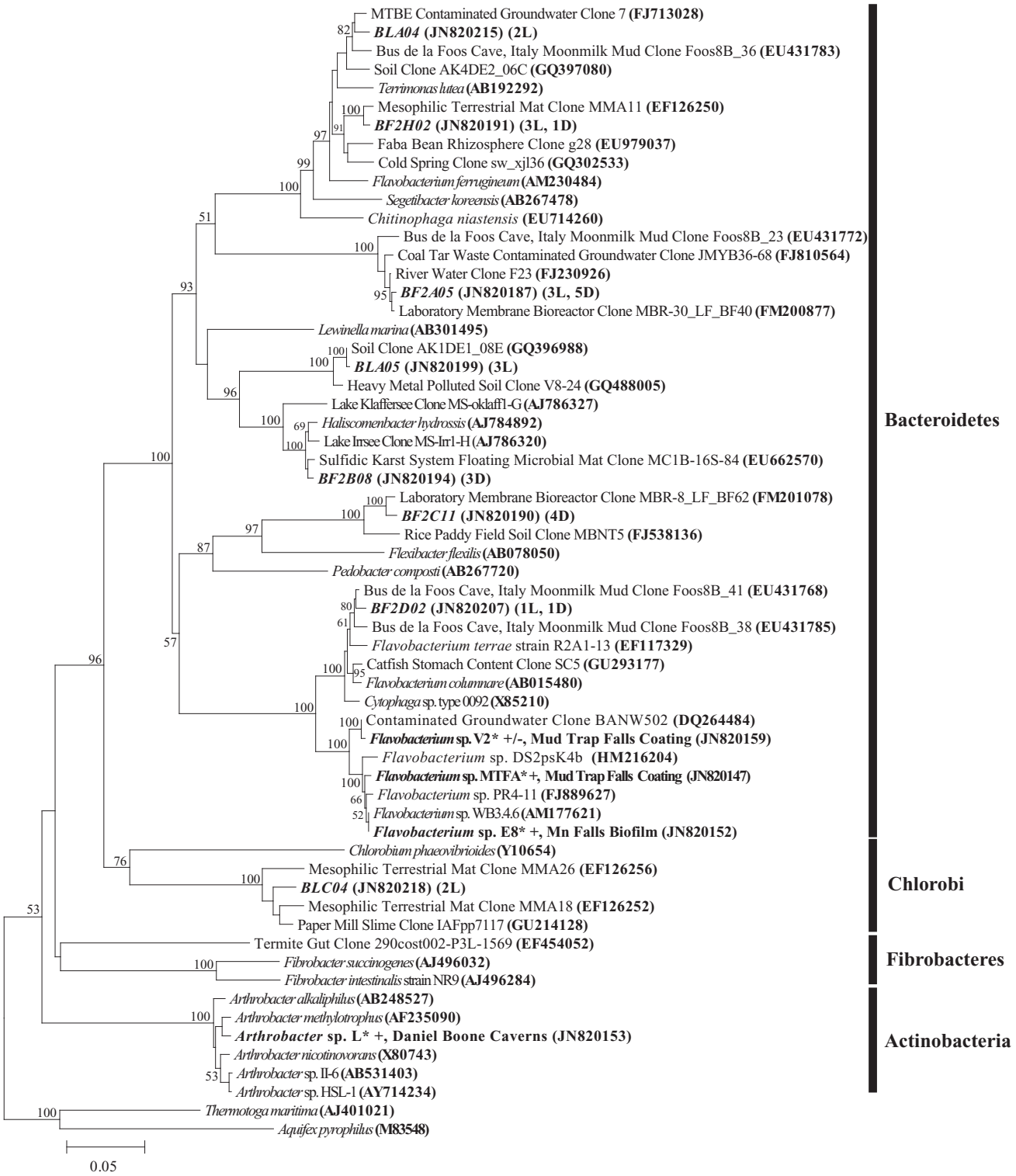

Fig. 7. Neighbor-joining tree inferring the phylogenetic relationship between ***cultured strains**** (asterisks) and those found in ***clone libraries*** in Carter Saltpeter Cave, Carter County, TN in this study for sequences clustering in the *Bacteroidetes*, *Chlorobi*, and *Actinobacteria*. Isolates that oxidize Mn(II) continually are indicated by a +; isolates that oxidize Mn(II) intermittently are indicated by a ±. Source of isolation is noted immediately before accession number. The number of sequences from each library [Mn Falls Light (L) and Mn Falls Dark (D)] representing a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position (see scale bar).

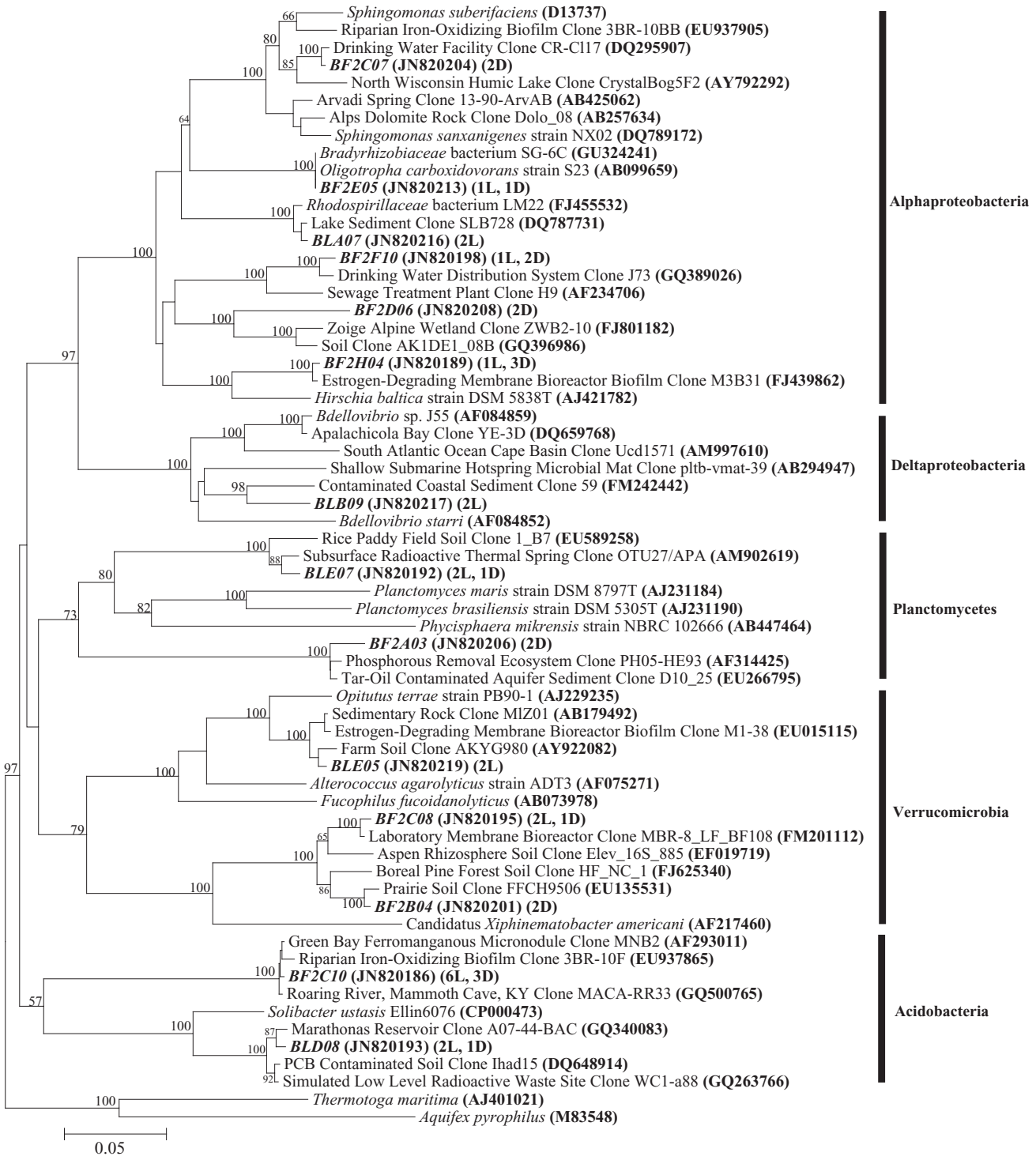

Fig. 8. Neighbor-joining tree inferring the phylogenetic placement of SSU rRNA gene sequences obtained from biofilms found in Carter Saltpeter Cave, Carter County, TN in this study for sequences clustering in the *Alpha*- and *Deltaproteobacteria* as well as *Planctomycetes*, *Verrucomicrobia*, and *Acidobacteria*. The number of sequences from each library [Mn Falls Light (L) and Mn Falls Dark (D)] representing a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position (see scale bar).

Table 3. Media used to isolate Mn(II)-oxidizing bacteria in this study

Media	Isolate	Phylogenetic Affiliation
Modified AY Media	*Arthrobacter* sp. L	*Actinobacteria*
Modified Burk's Nitrogen-free Media	*Pseudomonas* sp. N3	*Gammaproteobacteria*
	Pseudomonas sp. N4	*Gammaproteobacteria*
	Pseudomonas sp. T2	*Gammaproteobacteria*
	Pseudomonas sp. T4	*Gammaproteobacteria*
FMO2 (arabinose as a carbon source)	*Flavobacterium* sp. E8	*Bacteroidetes*
	Janthinobacterium sp. A6	*Betaproteobacteria*
FMO2 (casamino acids as a carbon source)	*Flavobacterium* sp. MTFA	*Bacteroidetes*
FMO2 (casamino acids as a carbon source with Gelrite gellan gum as a solidifying agent)	*Pseudomonas* sp. 9	*Gammaproteobacteria*
FMO2 (succinate as a carbon source)	*Leptothrix* sp. G6	*Betaproteobacteria*
	Pseudomonas sp. Mn Falls 11	
Nitrate mineral salts	*Acinetobacter* sp. V1	*Gammaproteobacteria*
	Flavobacterium sp. V2	*Bacteroidetes*

All media used agar as a solidifying agent, unless otherwise noted. Media recipes are described in detail in the methods.

1,485 bp alignment to the Mn-oxidizing, sheath-forming *Leptothrix cholodnii (*formerly *L. discophora)* SP-6 (Emerson and Ghiorse 1992), and it was obtained from a serial dilution culture containing 2.5×10^{-8} g wet weight deposit material. *Leptothrix*-like morphologies, as evidenced by straight, hollow sheaths coated in Mn and Fe, were detected in *in-situ* based glass slide incubations (Figures 10a and 10b) at the Mud Trap Falls seep and in SEM images of sample material from DBC's Top of V site (Figure 10c).

Two Mn(II)-oxidizing isolates obtained from ferromanganese deposits within CSPC clustered within the *Bacteroidetes* (Figure 7): *Flavobacterium* sp. E8 and

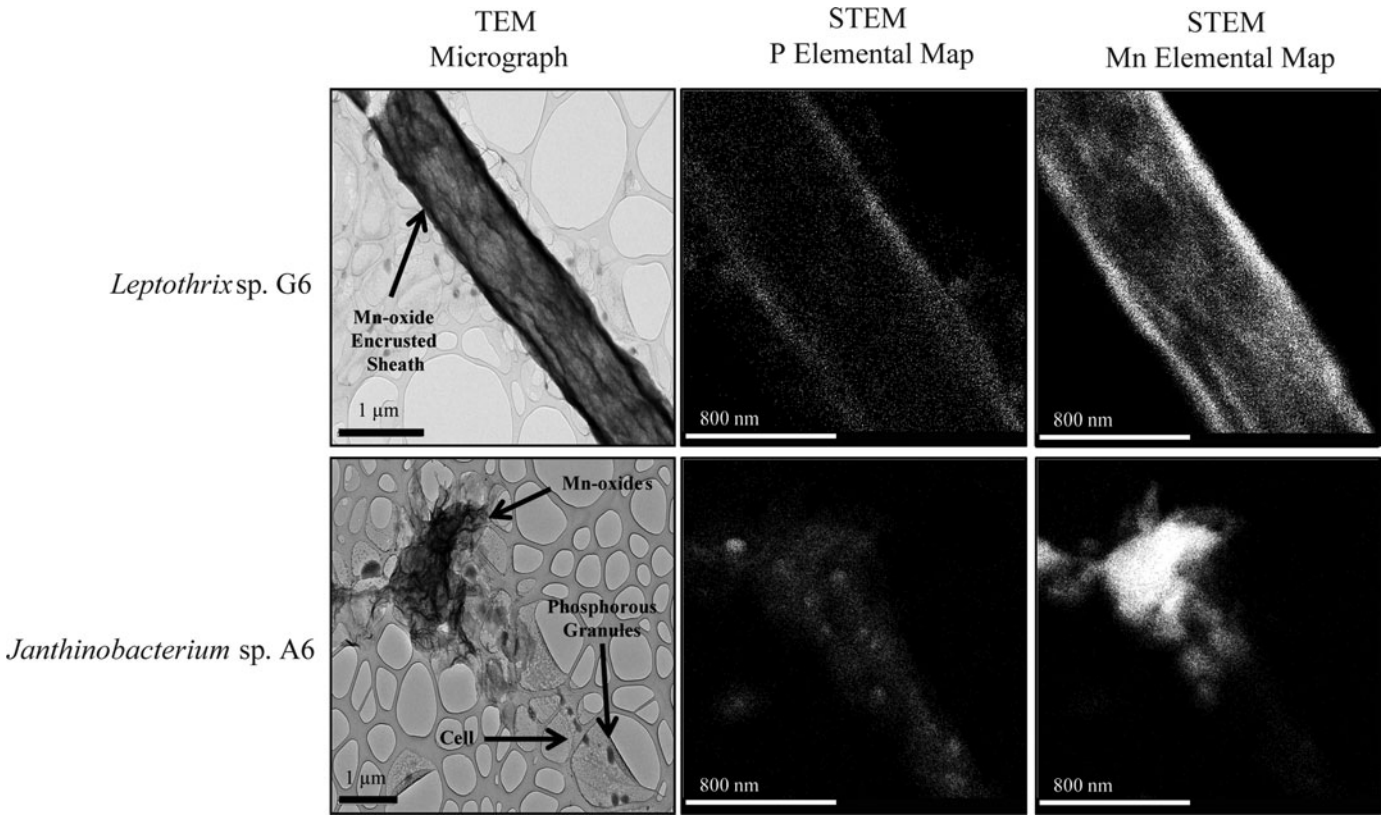

Fig. 9. TEM microscopy of the sheath-forming isolate *Leptothrix* sp. G6 and *Janthinobacterium* sp. A6. TEM micrographs (scale bars represent1 μm) demonstrated the presence of electron-dense Mn deposits associated with bacterial cells. STEM P and Mn elemental maps (scale bars represent 800 nm) revealed the location of phosphorous and manganese within bacterial cells.

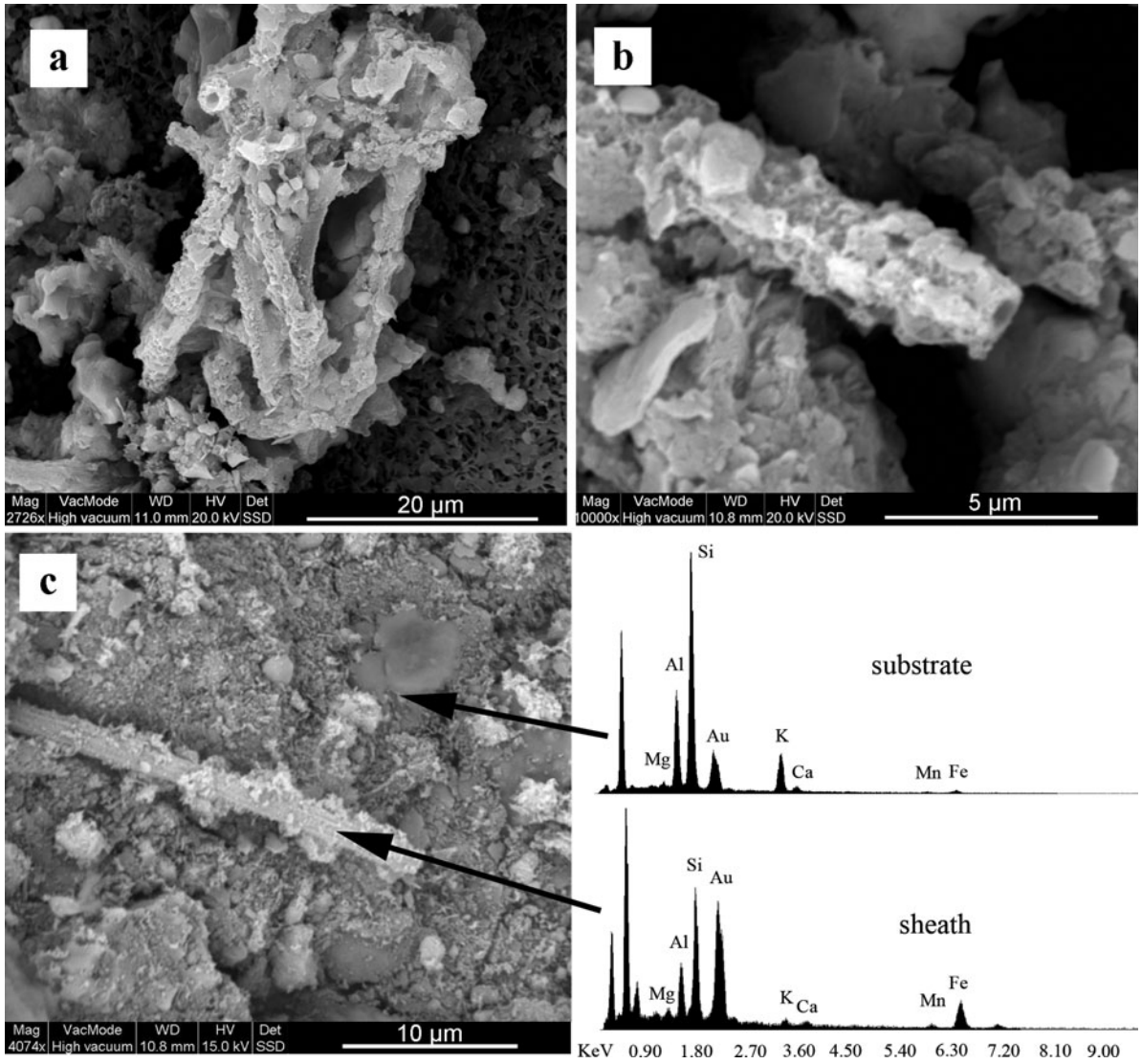

Fig. 10. SEM images (a,b) of a polycarbonate filter mounted on a glass slide and incubated for 6 months *in situ* at the top of the Mud Trap Falls seep (CSPC) revealed *Leptothrix*-like microbial morphologies. SEM micrograph of Daniel Boone Caverns Top of V site (c) revealed a putative *Leptothrix*; however, the hollow structure of the putative sheath was not verified. EDS spectra of the filament is enriched in Fe and Mn as compared to EDS spectra of the background material.

Flavobacterium sp. MTFA, which oxidize Mn(II) in liquid culture (Table 3). Members of this genus have previously been reported to oxidize Mn(II) (Ford and Mitchell 1990; Nealson 1978; Santelli et al. 2010). *Flavobacterium* sp. E8 is 98% identical over a 1,387 bp alignment to the Mn(II)-oxidizing *Flavobacterium* sp. DS2psk4b, which was isolated from coal mine drainage (Santelli et al. 2010). Isolate E8 was obtained from a serial dilution culture containing 2.5×10^{-10}g wet weight biofilm material.

A single isolate clustering within the *Actinobacteria*, *Arthrobacter* sp. L (Table 3, Figure 7), was obtained from a ferromanganese deposit located within Daniel Boone Caverns. *Arthrobacter* isolate L oxidizes Mn(II) in liquid culture; members of this genus have been reported to demonstrate this capability (Schweisfurth et al. 1978). *Arthrobacter* sp. L is 98% identical over a 1,457 bp alignment to its closest cultivated relative, *Arthrobacter methylotrophus*, a facultative methylotroph isolated from an enrichment culture containing dimethylsulfone as the sole source of carbon and energy (Borodina et al. 2000, 2002). Interestingly, in addition to Mn(II)-oxidizing microorganisms, two putative methylotrophic Mn(II)-oxidizers were isolated in this study: a member of the *Gammaproteobacteria*, *Acinetobacter* sp. V1 (Table 3, Figure 6), and a member of the *Bacteroidetes*, *Flavobacterium* sp. V2 (Table 3, Figure 7). Both isolates were capable of growth in liquid NMS media, which was designed to target methanotrophic Mn oxidizers by using methane as a sole carbon source. However, methanotrophy has not yet been confirmed in these strains.

Discussion

The Cave Geochemical Environment

Iron and manganese are the fourth and fifth most abundant elements in the Earth's crust, respectively (Edwards et al. 2004; Tebo et al. 2007), where Fe outweighs Mn by a ratio of ca. 58:1 in the upper continental crust (Turekian and Wedepohl 1961; Wedepohl 1995). The predominance of Fe over Mn in a variety of natural systems is well documented in studies of marine (Edwards et al. 2004; Nitahara et al. 2011) and freshwater systems (Johnson et al. 2012; Stein et al. 2001). However, in cases where the concentration of Mn is equal to or outweighs the concentration of Fe (Gradziński et al. 1995; Krumbein and Jens 1981) or where secondary mineral deposits are enriched in metal concentration relative to substrate geochemistry (Cunningham et al. 1995; Northup et al. 2003; Spilde et al. 2005, 2006), biomineralization processes are invoked as a causal factor in the formation of Mn-enriched geochemical environments.

These microbial biomineralization processes contribute to ferromanganese oxide accretion in this study, where Mn concentrations were enriched relative to bedrock concentrations and where the Mn:Fe ratio approached 1:1, such as Worley's Ribbon Rock and CSPC Mn Falls (Table 2). A Mn:Fe ratio of ca. 1:1 in cave ferromanganese deposits is a common ratio found in deep, oligotrophic systems in the southwest United States such as Lechuguilla and Spider Caves (Northup et al. 2003; Spilde et al. 2005). Previous research demonstrates the role of microsite geochemistry in establishing environmental niches (Engel et al. 2010; Macalady et al. 2008; Rossmassler et al. 2012), structuring microbial communities (Barton et al. 2007; Goldscheider et al. 2006; Shabarova and Pernthaler 2010), and influencing mineral precipitation (Frierdich et al. 2011) and composition (Post 1999; White et al. 2009) in subsurface karst systems. However, the role of microsite geochemistry on biomineralization in shallow, epigenic cave systems may be masked by agricultural, anthropogenic, and soil geochemical inputs.

Ferromanganese Biofilm Community Composition

In the present study, qPCR data indicated that bacterial cells represented on average 95% of the total microbial cells at sample sites. Most notably, the range of detection of bacterial cell numbers in our study (7.5×10^7 to 9.8×10^9 cells/mL) was two to four orders of magnitude higher than numbers typically reported within similar, pH neutral cave systems (Barton et al. 2006; Northup et al. 2000). In addition, Mn oxide-rich biofilm samples were estimated to contain 9.85×10^9 total microbial cells/g wet weight, a quantity that is two orders of magnitude greater than studies of ferromanganese deposits in Spider and Lechuguilla Caves (Spilde et al. 2005). Deep cave systems with limited energy supply and minimal human impact are considered to be oligotrophic environments, and recent research indicates that they are low-biomass environments with high levels of microbial diversity (Northup and Lavoie 2001; Barton et al. 2004; Boston et al. 2006; Hunter et al. 2004). The total cell count reported in the present study of surface influenced shallow caves is higher than that found in deeper, oligotrophic caves; however, it is not outside the range of numbers reported in studies of other environmental systems. Microbial cell abundance has been estimated to range from 1.29×10^9 to 7.6×10^{10} cells/mL in marine sediments (Nitahara et al. 2011), cold seep microbial mats (Grünke et al. 2011), arable soil (Torsvik et al. 2002), and filamentous microbial mats in sulfidic springs (Engel et al. 2004).

At the time of sampling, the CSPC Mn Falls site was thought to be heavily impacted by organic input from sewage effluent (Carmichael et al. 2013). Bacterial biomass in cave pools has been shown to increase proportionally with organic carbon input (Simon and Buikema Jr. 1997), and cell concentrations in the range of 1×10^9 cells/mL have been reported in sewage (Fierer and Lennon 2011). Therefore, high cell counts at sites within the CSPC system could be indicative of high levels of anthropogenic impact (e.g., nutrient loading), due in part to the shallow, surface-influenced depths of these caves. In fact, clone library data corroborate this idea as many of the clones obtained here were related to sequences obtained from fecal contaminated environments (see Figure 6, OTUs BF2B07, BF2E04, BF2F03, BLD10, and BLB01). However, it is important to note that if the environment has selected for microbes adapted to high nutrient loading, it is likely that many of these organisms may have above average SSU rRNA operon copy numbers (Klappenbach et al. 2000). Therefore, it is possible that the total cell number has been overestimated.

In general, a high degree of diversity within systems is supported by microbial metabolic versatility (Whitman et al. 1998) and the development of mutualistic associations in biofilm communities leading to the interdependency of organisms within the community (Fierer and Lennon 2011). The SSU rRNA data from the present study are suggestive of the presence of a variety of microbial metabolic strategies within the CSPC Mn Falls and Mud Trap Falls communities, as clone sequences obtained in this study are closely related to methanogens (OTU G01, Figure 5), hydrocarbon degraders (OTU BF2E03, Figure 6), ammonia-oxidizers (OTU BF2F03, Figure 6), denitrifiers (OTU BLB01, Figure 6), and iron or manganese-oxidizers. Several lines of evidence from the SSU rRNA molecular-based survey of CSPC suggest that metal-oxidation plays an important role in the formation of ferromanganous biofilms within the cave system.

Members of the *Proteobacteria* (47% of the sequences represented by dominant OTUs in the present study) are common constituents of cave clone libraries and have been detected in RNA-based surveys as metabolically active members of cave microbial consortia (Portillo et al. 2008). Molecular work from the present study indicates that *Leptothrix* are present in detectable numbers within the CSPC Mn Falls biofilm community. In fact, 6% of the total library sequences (from both libraries) were represented by OTU BF2A07 (Figure 6), suggesting that both samples included live cells from the growing edge of the *Leptothrix* sheaths (Fleming et al. 2011). Additionally, the organisms representing these OTUs were phylogenetically related (99% identity over a 1,489 bp sequence alignment) to the *Leptothrix* strain isolated from a high dilution suggesting that they play an important role in the biomineralization of Mn in southern Appalachian cave systems. *Leptothrix* spp.

are known to be capable of both Fe and Mn oxidation (Spring 2006; van Veen et al. 1978), and *in situ* slides of *Leptothrix* from CSPC Mud Trap Falls demonstrate both Mn and Fe oxidation, thus confirming the role of microbes in the formation of cave ferromanganese biofilms and crusts.

Although based on small sample size, molecular analyses indicate that the most abundant and detectable populations in CSPC ferromanganese deposits (e.g., *Leptothrix*, *Pseudomonas*, and *Flavobacterium*-related organisms) are distinct from the populations identified by Northup et al. (2003) in a survey of the microbial communities inhabiting ferromanganese deposits in Lechuguilla and Spider Caves, which included taxa related to *Hyphomicrobium*, *Pedomicrobium*, *Leptospirillum*, *Stenotrophomonas*, and *Pantoea*-related organisms. These two distinct microbial communities may exhibit a level of functional redundancy in Fe- and Mn-biomineralization capacity within the two cave systems. Evidence from this study indicates that shallow cave systems may harbor Mn-oxidizing consortia with a unique signature in comparison to other cave systems, although deeper sequencing efforts may reveal more overlap.

Questions exist regarding the nature of these community differences, and whether these differences relate to differences in nutrient input in anthropogenically impacted vs. pristine cave systems, or simply the fact that these two cave systems differ in both regional geology and hydrology. Lechuguilla Cave has been described as deep and oligotrophic (Northup et al. 2003), whereas a parallel study of CSPC indicates that it has been severely impacted by nutrient loading from the surface (Carmichael et al. 2013). Future research should attempt to elucidate the cause of these observed differences, with an initial focus on the increased role of surface impact in shallow vs. deep cave systems.

Archaea, like Bacteria, are ubiquitous within the environment (DeLong 1992) and play a key role in the maintenance of biogeochemical cycles (Goldscheider et al. 2006). Yet, Archaea remain understudied members of microbial communities, particularly in caves. The role of Archaea in the formation and transformation of cave ferromanganese deposits is debatable, although molecular evidence of Archaea in clone libraries generated from cave ferromanganese deposits (Northup et al. 2003) has led some investigators (Tebo et al. 2005) to speculate regarding the possible existence of a new class of Archaea capable of Mn-biomineralization.

In the present study, Archaea represented ca. 5.0% of the total microbial population in a cave biofilm and ferromanganese coating. Archaeal community diversity was relatively low in comparison to bacterial community diversity; however, this trend is consistent with some cave systems (Macalady et al. 2007; Legatzki et al. 2011), marine ferromanganese crusts (Nitahara et al. 2011), and some other environments (DeLong 1992). The archaeal community in this study was dominated by members of Thaumarchaea, primarily Marine Group 1 OTU TDO2. This group accounted for 37% of the total archaeal diversity in the library, and 92% of the Thaumarchaeal diversity. At least one clone from the SAGMA groups 1 and 2 of the Thaumarchaea was also recovered and this clone (TG02) shared 92% identity to one recovered from Lechuguilla Cave, clone CV1B4 (Northup et al. 2003, and Figure 4). Members of the Rice Cluster V and Deep Sea Hydrothermal Vent Group 6 (DSHV6) Euryarchaea were the second and third most dominant archaeal groups, representing 32% and 29% respectively of the total archaeal diversity captured in this study.

Of the six DSHV6 OTUs identified in this study, four (FD02, FF04, TD04, and TD06) were less than 95% similar to their closest relatives as determined by BLAST analysis. This high degree of divergence (Amann et al. 1995) indicates that these sequences represent novel lineages unique to CSPC or other similar systems. Several findings emphasize the need to ascertain the functional role of Archaea in cave biogeochemical cycling: 1) the dominance of sequences related to Thaumarchaeal OTU TD02 in the CSPC Mn(II)-oxidizing biofilm community, 2) recent evidence from other studies of metabolically active Thaumarchaea in similar cave systems (Gonzalez et al. 2006), and 3) the recovery of several novel Euryarchaeal lineages in CSPC.

Mn-biomineralization in Cave Ferromanganese Deposits

The present study is significant due to the broad taxonomic array of bacteria isolated from cave ferromanganese deposits that demonstrate the ability to oxidize Mn(II) in culture. The isolation of a *Janthinobacterium* sp. capable of Mn(II)-oxidation represents the first report of a Mn(II)-oxidizing member from a cave, although members of this genus have been both detected in clone libraries, as well as isolated from desert varnish (Northup et al. 2010). This finding expands the phylogenetic diversity of known cave Mn-oxidizers to include new genera and provides further support for the importance of this process to the microbial cell. In addition, this study represents the first report of cave isolates among the genera *Flavobacterium* and *Arthrobacter* that also demonstrated Mn-biomineralization capacity *in vitro*. *Flavobacterium* (Ikner et al. 2007) and *Arthrobacter* (Ikner et al. 2007; Laiz et al. 2000) species have been isolated from cave systems in prior studies, although the Mn-biomineralization capacity of these isolates was not established.

Iron and manganese oxidizing *Leptothrix* spp. are commonly isolated from freshwater systems and are particularly abundant at redox interfaces (Spring 2006; van Veen et al. 1978). However, *Leptothrix* spp. typically lose their sheath-forming capacity in culture, so isolation of a close relative of the sheath-forming strain SP-6 from a high dilution is unusual and noteworthy. The isolation of a Mn(II)-oxidizing *Leptothrix* from CSPC represents the first reported isolation of this organism from a cave in over twenty years (Moore 1981; Peck 1986), although recent work has demonstrated the presence of *Leptothrix*-like morphologies in caves using scanning electron microscopy (de los Ríos et al. 2011; Florea et al. 2011; Frierdich et al. 2011). In-situ based cultivation efforts from this study (Figures 10a and 10b) demonstrate that *Leptothrix* spp. are actively oxidizing Mn in the field, corroborating *in-vitro* cultivation results. Combined data from this and other studies suggests that *Leptothrix* spp. may be widespread in epigenic cave systems. Further, results demonstrate that Mn-oxidizing

Leptothrix spp. are particularly abundant and active in our study sites.

Over 30 different metal oxide and hydroxide minerals can be found in caves (Hill and Forti 1997) though it is important to note that not all black or dark brown deposits in caves are Mn oxides (Gázquez et al. 2012; Hill 1982) and it is unlikely that all are formed by microbes. However, Mn-oxidizing microbes have been shown to produce several of the Mn oxide minerals (such as birnessite and todorokite) commonly found in caves (Frierdich et al. 2011; Gadd 2007; Northup and Lavoie 2001; Santelli et al. 2010; Spilde et al. 2005). The isolation of three Mn(II)- biomineralizing organisms from high dilutions (2.5×10^{-8} g wet weight and 2.5×10^{-10} g wet weight) is suggestive of the environmental relevance of these genera (*Janthinobacterium*, *Leptothrix*, and *Flavobacterium*) in the formation of cave ferromanganese deposits in the upper Tennessee River Basin. The diversity of isolates with inferred biomineralization capacity discovered in this study, combined with evidence of *in situ* Mn-oxidation by *Leptothrix* spp. demonstrates the importance of these organisms in cave biogeochemical cycles.

Conclusions

The present study represents the first geomicrobiological analysis of ferromanganese deposits within the cave-rich, yet understudied southern Appalachian karst system. SSU rRNA based molecular surveys at Mn-enriched sites reveal the genetic potential for Mn-biomineralization within these deposits. Biomineralization capacity was inferred via culture-dependent surveys, which resulted in the isolation of a broad taxonomic array of Mn(II)-oxidizing bacteria from cave ferromanganese deposits. Combined molecular, cultivation-based, and *in situ*-based evidence demonstrate that Mn(II)-oxidizing bacteria are abundant and environmentally relevant species in cave ferromanganese deposits. Further, at the present sequencing depth, results suggest that microbial communities contributing to the accretion of ferromanganese deposits in these shallow cave systems share little taxonomic similarity with those in deeper cave systems. The Mn-oxidizing bacteria identified here likely play a role in mediating cave biogeochemical cycles, forming and transforming the cave mineral environment, and are vital contributors (via functional diversity) to the maintenance of cave microbial consortia (Warren and Kauffman 2003) within these fragile and unique cave systems.

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